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I, KIM MARSHALL , MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification as filed on 7 March 1997 in connection with Application No. PO 5494 for a patent by DARATECH PTY LTD and PIG RESEARCH AND DEVELOPMENT CORPORATION.

I further certify that the name of the applicant has been amended to AGRICULTURE VICTORIA SERVICES PTY. LTD. and PIG RESEARCH AND DEVELOPMENT CORPORATION pursuant to the provisions of Section 104 of the Patents Act 1990.

I further certify that the annexed specification is not, as yet, open to public inspection.

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day of April 1998

KIM MARSHALL
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Regulation 3.2

Agriculture Victoria Services Pty Ltd. ~~Daratech Pty Ltd~~

AND

Pig Research and Development Corporation

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Novel bacterial pathogens"

The invention is described in the following statement:

NOVEL BACTERIAL PATHOGENS

The present invention relates generally to novel isolated species of pathogenic bacteria and to immunoreactive molecules which are derived therefrom and their use in vaccine preparations. The vaccine preparations of the present invention are useful in protecting host organisms against bacterial infections. More particularly, the present invention is directed to an isolated species or serovar of bacteria belonging to the genus *Leptospira*. The present invention is further directed to methods of detection, identification and quantification of a *Leptospira* species or serovar.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Bacteria of the genus *Leptospira* are either pathogenic or saprophytic spirochaetes comprising several known species (Pathogenic: *L.interrogans, L.inadai, L.borgpetersenii, L. santarosai, L. kirschneri, L. weilii* or *L. noguchii* ; Saprophytic: *L.biflexa, L. meyeri* or *L. wolbachii*), each of which comprises a large number of serovars. Saprophytic serovars of *Leptospira* are omnipresent in fresh surface waters and occasionally found in sea water. Pathogenic *Leptospira* serovars occur naturally in a large variety of livestock animals, companion animals, wild animals and humans. The host range of *Leptospira* serovars is generally quite broad, however the bacterium may produce differing symptoms in each host organism which it has infected.

In a primary (maintenance) host in which a pathogenic *Leptospira* serovar is maintained, reproductive disease is typical. Alternatively, infection may be asymptomatic. Pathogenic *Leptospira* serovars may also cause acute, febrile, systemic disease in mammals. Acute

febrile disease is also characteristic of many human infections.

In livestock animals such as pigs and possibly horses and dogs or other species, the pathogen *L.interrogans* serovar *bratislava* causes reproductive disease leading to infertility, abortions or stillbirth and has been cited as a possible causative agent of seasonal infertility (Chappel *et al.*, 1993; Ellis *et al.*, 1983; Ellis *et al.*, 1985; Ellis *et al.*, 1986a,b; Frantz *et al.*, 1988). Infection with *L.interrogans* serovar *bratislava* is endemic in European and North American swine herds. In Australian swine herds, the pathogenic serovars *L.interrogans* serovar *pomona* and *L. borgpetersenii* serovar *tarassovi* have long been recognised (Chappel *et al.*, 1987a,b; Chappel *et al.*, 1990; Davos, 1977), however many Australian herds have also tested positive for the presence of *L.interrogans* serovar *bratislava* using the microscopic agglutination test, hereinafter referred to as "MAT" (Chappel *et al.*, 1992; Chappel *et al.*, 1993). *Leptospira interrogans* serovar *bratislava* is notoriously recalcitrant to standard isolation techniques, using samples from the infected host organism as starting material. This factor has to date prevented the preparation in Australia of vaccines which protect animals specifically against infection by serovar *bratislava.*

In work leading up to the present invention, the inventors sought to produce vaccines and diagnostic agents for pathogenic *Leptospira*, by isolating *Leptospira* serovars, in particular *L.interrogans* serovar *bratislava* from swine herds with MAT titres to serovar *bratislava* and with immunochemical evidence of leptospiral infection. Surprisingly, a novel leptospire was isolated which does not cross-react in MATs with other pathogenic serovars including serovars *bratislava, pomona* and *tarassovi.* The new species of *Leptospira*, and recombinant nucleic acid, polypeptides or immunoreactive molecules which are derived therefrom, and derivatives, homologues or analogues thereof, provide the means to develop a range of diagnostic and therapeutic agents for *Leptospira* infection which were hitherto not available.

Accordingly one aspect of the present invention provides an isolated pathogenic *Leptospira* bacterium or derivative serovar thereof which grows at temperatures of 13°C

to 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

In an even more preferred embodiment, said bacterium is further capable of infecting a livestock or companion animal, in particular a livestock or companion animal selected from the list comprising pigs, cattle, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

In a most preferred embodiment, said pathogenic *Leptospira* bacterium is capable of infecting said animal and inducing reproductive disease therein.

The term "reproductive disease" as used herein shall be taken to refer to any abnormality of the reproductive system of a livestock animal, in particular pigs or cattle which reduces the fecundity of said animal, for example an abnormality characterised by infertility of said animal including seasonal infertility or abnormal development of a foetus in said animal or spontaneous abortion of a foetus in said animal or failure to conceive by said animal. In the context of the present invention, such reproductive disease is caused by infection of a livestock animal, in particular pigs or cattle, with a pathogenic bacterium of the genus *Leptospira*, in particular leptospiral serovar *hurstbridge* or a derivative serovar thereof.

In an alternative embodiment, the present invention provides an isolated *Leptospira* bacterium or derivative serovar thereof which contains genetic sequences from nucleotide position 51 to nucleotide position 199 of the 165 ribosomal RNA (rRNA) gene which are at least 85% identical to the rRNA genetic sequences of *Leptospira inadai* serovar *lyme* and less than 80% identical to the rRNA genetic sequences of *Leptospira biflexa* serovar *patoc*, wherein said pathogenic bacterium is capable of growing at temperatures of 13°C to 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and

even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

In a preferred embodiment, the *Leptospira* bacterium or derivative serovar of the present invention is further characterised in being a pathogenic bacterium.

More preferably, said pathogenic bacterium is further capable of infecting a livestock animal, in particular a livestock animal selected from the list comprising pigs, cattle, sheep, goats, horses, deer, alpacas or a companion animal such as a dog or cat, amongst others.

According to this embodiment of the invention, wherein a pathogenic *Leptospira* bacterium infects said livestock animal, it is most preferred that said bacterium induces reproductive disease therein.

In another alternative embodiment of the present invention, there is provided an isolated *Leptospira* bacterium or derivative serovar thereof which contains genetic sequences which are at least 80% identical to the nucleotide sequence set forth in SEQ ID NO:1 or its complement, or a derivative, homologue or analogue thereof.

According to this embodiment, it is preferred that said pathogenic *Leptospira* bacterium is further capable of growing at temperatures of 13°C to 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

It is also preferred that the percentage identity to the nucleotide sequence set forth in SEQ ID NO:1 is at least 80%. According to this embodiment of the invention, it is more preferred that the genetic material of said pathogenic *Leptospira* bacterium or derivative serovar thereof be at least 90% identical to SEQ ID NO:1, even more preferably at least 95% identical and still more preferably at least 99% identical including 100% identical.

In a further alternative embodiment, the present invention provides an isolated pathogenic *Leptospira* bacterium or derivative serovar thereof which contains genetic material capable of hybridising under high stringency conditions to the nucleotide sequence set forth in SEQ ID NO:1 or its complementary nucleotide sequence, or a derivative, homologue or analogue thereof.

Preferably, said genetic material is selected from the list comprising RNA or DNA.

More preferably, the present invention provides an isolated pathogenic *Leptospira* bacterium or derivative serovar thereof which contains RNA or DNA capable of hybridising under high stringency conditions to the nucleotide sequence set forth in SEQ ID NO:1 or its complementary nucleotide sequence, or a derivative, homologue or analogue thereof wherein said bacterium is further capable of growing at temperatures of 13°C to 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

For the purposes of defining the level of stringency, a high stringency is defined herein as being a hybridisation and/or a wash thereafter carried out in 0.1xSSC buffer, 0.1% (w/v) SDS at 65°C. Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification, (to parameters affecting hybridisation between nucleic acid molecules), reference is found in pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

In a particularly preferred embodiment of the present invention, there is provided an isolated bacterium or derivative serovar which:

1. Is a pathogenic species belonging to the genus *Leptospira*;

2. Grows at temperatures in the range of 13°C to 37°C;

3. Grows in media containing at least 225µg/ml 8-azaguanine;

4. Is capable of infecting a livestock or companion animal, in particular a livestock or companion animal selected from the list comprising pigs, cattle, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others;

5. Is capable of inducing reproductive disease as hereinbefore defined in at least one of said infected animal; and

6. Contains a genetic sequence which comprises a sequence of nucleotides, or is complementary to a genetic sequence which comprises a sequence of nucleotides which is at least 80% identical to the nucleotide sequence set forth in SEQ ID NO:1 or a derivative, homologue or analogue thereof.

In connection with this invention, said *Leptospira* bacterium has been deposited pursuant to and in satisfaction of, the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, and are made with the Australian Government Analytical Laboratories (AGAL), 1 Suakin Street, Pymble, New South Wales 2073, Australia (Postal Address: PO Box 385 Pymble NSW 2073 Australia) on 15 November, 1995 and accorded AGAL Accession Number N95/69684.

In a most particularly preferred embodiment, the present invention provides an isolated pathogenic *Leptospira* bacterium or derivative serovar which possesses the characteristics or attributes of the microorganism deposited with AGAL under AGAL Accession Number N95/69684 or is within the same serogroup (as defined by Faine, 1994) as the microorganism N95/69684 or is in the same species as the microorganism N95/69684 or is immunologically cross-reactive with the microorganism N95/69684.

For the purposes of nomenclature, the isolated pathogenic *Leptospira* bacterium deposited under AGAL Accession Number N95/69684 has been designated as leptospiral serovar *hurstbridge*. It will be known to those skilled in the art that certain characteristics as hereinbefore defined for leptospiral serovar *hurstbridge* may vary between serovars, while other characteristics will remain constant between serovars

belonging to the same species. For the purposes of the present invention a serovar of leptospiral serovar *hurstbridge* is defined herein as a pathogen of a livestock or companion animal, in particular a livestock or companion animal selected from the list comprising pigs, cattle, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others which is antigenically-related and/or genetically-related to the leptospiral serovar *hurstbridge* deposited under AGAL Accession Number N95/69684. The present invention encompasses all such leptospires.

A "derivative" of the leptospiral serovar *hurstbridge* is a bacterium which has been developed by mutation, recombination, conjugation or transformation of leptospiral serovar *hurstbridge* as hereinbefore defined. Preferably, a derivative serovar of leptospiral serovar *hurstbridge* is serologically cross-reactive or immunologically cross-reactive with the leptospiral serovar *hurstbridge* defined herein, in particular the leptospiral serovar *hurstbridge* assigned AGAL Accession Number N95/69684. It will be known to a person skilled in the art how to produce derivative serovars of leptospiral serovar *hurstbridge*.

A further embodiment of the present invention provides an isolated serovar of leptospiral serovar *hurstbridge* as hereinbefore defined or a derivative serovar thereof. Preferably, said serovar of a leptospiral serovar *hurstbridge* is genetically-cross-reactive or immunologically-cross-reactive with the serovar deposited with AGAL under Accession No. N95/69684 on 15 November, 1995.

More preferably, said serovar is identical to the serovar deposited with AGAL under Accession No. N95/69684 on 15 November, 1995.

According to this embodiment of the invention, said isolated serovar may be determined to be immunologically-cross-reactive or genetically-cross-reactive or genetically-cross-hybridising with the serovar of the leptospiral serovar *hurstbridge* deposited under AGAL Accession No, N95/69684 by any means known to those skilled in the relevant art, including, but not limited to, serological, immunological, or molecular-biological

means. Serological means include MAT titre estimations (Cole et al., 1973; Chappel, 1993a). Immunological means include ELISA, Western blot immunoelectrophoresis, immunodiffusion techniques, rocket gel electrophoresis, radio-immunoassay techniques, amongst others. Molecular-biological means include nucleic acid hybridisation, nucleic acid sequencing techniques, polymerase chain reaction techniques and variations thereto, amongst others. Those skilled in the relevant art will be aware of variations and optimisations which may be applied to these procedures, in typing the leptospiral serovar *hurstbridge*.

The invention described according to this aspect extends to said isolated bacterium when provided as a culture in liquid or solid form, such as but not limited to a glycerol stock, stab, slope, plate or in a freeze-dried or otherwise-dried form, for example on a membraneous filter or paper disc.

A second aspect of the present invention is directed to a method of isolation of the pathogenic *Leptospira* serovar *hurstbridge* or an immunologically-cross-reactive or a genetically-cross-reactive serovar or a derivative serovar thereof comprising the steps of:

1. Collection of animal tissue from a host organism which is infected with said pathogen;
2. Homogenisation of said tissue in homogenisation medium suitable for maintaining the integrity of said pathogenic bacterium; and
3. Culture of said tissue containing said *Leptospira* bacterium in a culture medium for a time sufficient to allow bacteria to grow to the required density.

The culture medium may be any medium appropriate for the purpose of culturing a *letospira* bacterium, which are generally known to those skilled in the art, for example EMJH medium described by Chappel (1993b).

According to this aspect of the present invention, a person skilled in the art would be aware that said culture of *Leptospira* may require sub-culturing at certain intervals, in

order to maintain the viability of the culture. Such sub-culturing serves to replace nutrients in the media which are essential to viability and/or growth of the bacterium. If sufficient cycles of sub-culturing are carried out, this will eventually produce a bacterial culture which is essentially free of contaminating animal tissue derived from the host organism.

Preferably, the animal tissue from which said pathogen is obtained is blood or tissue of the urogenital tract selected from the list comprising bladder, kidney, uterus or fallopian tube, testes or ovaries or, alternatively, from liver or lung tissue, or from body fluids or exudates such as urine or cerebrospinal fluid, amongst other sources. More preferably, said tissue originates from a preferred host of the pathogenic leptospiral serovar *hurstbridge*, in particular a livestock or companion animal selected from the list comprising pigs, cattle, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

It will be understood by those skilled in the art that there are a range of suitable homogenisation media which may be used, the only requirement being that the particular homogenisation medium used maintains the bacterium in a viable state such that sufficient viable cells exist in the homogenate to establish a viable culture.

The present invention extends to the use of any suitable homogenisation medium in the isolation of the pathogenic leptospiral serovar *hurstbridge* including, for example, media containing phosphate-buffered albumin.

Preferably, the culture medium contains in addition to 8-azaguanine, 5-fluorouracil and at least one antibiotic selected from the list comprising a rifamycin, macrolide polyene or quinoline antibiotic, amongst others.

Rifamycin antibiotics are high substituted macrocyclic compounds which are active against Gram-positive bacteria and certain Gram-negative bacteria but to which spirochaete bacteria including *Leptospira* bacteria are resistant. Rifamycins specifically inhibit eubacterial DNA-dependent RNA polymerase, binding to the β-subunit and

inhibiting transcription.

The macrolide polyenes are characterised by a substituted or unsubstituted lactone ring containing a rigid, lipophilic region of unsubstituted *trans*-conjugated double bonds and a flexible, hydrophilic hydroxylated region. Macrolide polyenes interact with sterols in the cytoplasmic membrane, causing leakage of ions and small molecules. Macrolide antibiotics are not effective against bacteria which do not contain sterols in their membranes. Macrolide antibiotics are microbistatic at low concentrations or microbicidal at higher concentrations against yeast and other fungi and against protozoa which contain sterols in their membranes. Preferred macrolide polyenes are selected from the list comprising amphotericin, aureofungin, candicidin B, etruscomycin, filipin, hamycin, hystatin, perimycin, pimaricin and trichomycin amongst others.

Quinoline antibiotics contain a substituted 4-quinoline ring and are primarily active against Gram-negative bacteria. Preferred quinoline antibiotics include but are not limited to antibiotics selected from the list comprising naladixic acid, cinoxacin, oxolinic acid, pipemidic acid, ciprofloxacin, enoxacin, norfloxacin, ofloxacin or perfloxacin, amongst others.

The list of antibiotics provided for the isolation of a *Leptospira* bacterium according to the present invention is not exhaustive and the person skilled in the art will appreciate that alternative or additional antibiotics may be used. The person skilled in the art will also be aware that the animal tissue from which the pathogenic leptospire is to be isolated may contain several contaminating microorganisms in addition to said *Leptospira* bacterium and the particular combination of antibiotics selected for use will vary depending upon the nature of the contaminating microorganisms present. The present invention clearly contemplates the use of additional antibiotics in the culture media used for the isolation of said pathogenic *Leptospira* bacterium.

In a particularly preferred embodiment, the present invention provides a method of isolation of a pathogenic *Leptospira* bacterium as hereinbefore described wherein said

bacterium is a serovar which has been deposited with AGAL on 15 November, 1995 and assigned AGAL Accession Number N95/69684.

In a particularly preferred embodiment, said method is useful for the isolation of a pathogenic *Leptospira* bacterium or derivative serovar thereof wherein said bacterium is leptospiral serovar *hurstbridge*, more preferably the serovar deposited with AGAL on 15 November, 1995 and assigned AGAL Accession Number N95/69684.

A third aspect of the present invention provides agents and chemical compositions for use in the isolation of the pathogenic leptospiral serovar *hurstbridge* or an immunologically-cross-reactive serovar or genetically-cross-reactive serovar or a derivative serovar thereof, essentially according to the methods described herein.

In a preferred embodiment, the present invention provides agents and chemical compositions for use in the isolation of a pathogenic *Leptospira* bacterium in particular the leptospiral serovar *hurstbridge* or a derivative serovar thereof according to the methods described herein, wherein said agent or chemical composition is a culture medium for the selective growth of said bacterium.

According to this aspect of the present invention, said agent or chemical composition may be in powdered, liquid, tablet, pellet, capsule or other form.

The present invention extends to an agent or chemical composition as described herein, wherein said agent or chemical composition is used for, or intended to be used for the isolation, detection, purification, culture or storage of a pathogenic microorganism, preferably a pathogenic bacterium, more preferably a pathogenic *Leptospira* bacterium in particular the leptospiral serovar *hurstbridge* such as the serovar deposited under AGAL Accession Number N95/69684 or a derivative serovar thereof.

A fourth aspect of the present invention provide an isolated nucleic acid molecule comprising a sequence of nucleotides which corresponds to, or is complementary to a

sequence of nucleotides which corresponds to the 16S rRNA gene or a derivative, homologue or analogue thereof of a pathogenic *Leptospira* bacterium, wherein said bacterium is capable of growing at temperatures of 13°C to 37°C and/or in the presence of at least 100μg/ml 8-azaguanine, preferably at least 150μg/ml 8-azaguanine, more preferably at least 200μg/ml 8-azaguanine and even more preferably at least 250μg/ml, or up to and including a concentration of 500μg/ml 8-azaguanine.

In a particularly preferred embodiment, said pathogenic *Leptospira* bacterium is the leptospiral serovar *hurstbridge*, more particularly the serovar deposited with AGAL on 15 November, 1995 under AGAL Accession No. N95/69684 or a derivative serovar thereof.

Reference herein to "genes" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5′- and 3′- untranslated sequences); or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5′- and 3′-untranslated sequences of the gene.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product. Preferred rRNA genes may be derived for a naturally-occurring serovar, in particular the rRNA gene of leptospiral serovar *hurstbridge*, by standard recombinant techniques. Generally, a rRNA gene may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or additions. Nucleotide insertional derivatives of a rRNA gene of the present invention include 5′ and 3′ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional nucleotide sequence variants are those in which one or more nucleotide are introduced into a predetermined site in the nucleotide sequence although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more nucleotides from the sequence. Substitutional nucleotide variants are those in which at least one

nucleotide in the sequence has been removed and a different nucleotide inserted in its place.

For the present purpose, homologues of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as, or at least 80% identical to, a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence within said sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements.

Analogues of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated nucleic acid molecule, for example carbohydrates, radiochemicals including radionucleotides, reporter molecules such as, but not limited to DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

Derivatives of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains significant sequence similarity to said sequence or a part thereof. Generally, the nucleotide sequence of the present invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions. Nucleotide insertional derivatives of the nucleotide sequence of the present invention include 5′ and 3′ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of said sequence, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional variants are characterised by the removal of one or more nucleotides from the nucleotide sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place.

For the purposes of the present invention, it is preferred that the nucleic acid molecule of the invention is the 16S rRNA genetic sequence of the leptospiral serovar *hurstbridge* which has been deposited with AGAL under Accession Number N95/69684. It will be known to those skilled in the relevant art that derivative serovars of the leptospiral serovar *hurstbridge*, or serovars within the same species will contain 16S rRNA genetic sequences which are more closely related to the 16S rRNA of serovar *hurstbridge* than are the 16S rRNA genetic sequences obtained from more distantly-related, or unrelated serovars of *Leptospira*. As a consequence, the genetic sequence of the present invention is at least useful in determining whether or not a pathogenic *Leptospira* bacterium is closely related to serovar *hurstbridge*. Said genetic sequence is also useful in the isolation of genetic sequences from serovars of *Leptospira* which are closely-related to leptospiral serovar *hurstbridge*.

The person skilled in the art will be aware of nucleic acid hybridisation techniques techniques which may be used to identify leptospires which are related to serovar *hurstbridge*, in particular the various hybridisation stringencies which may be employed in such an identification procedure. For the purposes of the defining the level of stringency, a low stringency is defined herein as being a hybridisation and/or wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at 28°C. Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification, of the parameters affecting hybridisation between nucleic acid molecules, reference is found in pages 2.10.8 to 2.10.16 of Ausubel *et al* (1987), which is herein incorporated by reference.

The person skilled in the art will appreciate that the nucleic acid molecules of the present invention may correspond to the naturally-occurring sequence or may differ by one or more nucleotide substitutions, deletions and/or additions. Accordingly, the present invention extends to rRNA genes and any genes, mutants, derivatives, parts, fragments, homologues or analogues thereof which are at least useful as, for example,

genetic probes, or primer sequences in the enzymatic or chemical synthesis of said gene, or in the generation of immunologically interactive recombinant molecules, or in the isolation or detection of a pathogenic *Leptospira* bacterium.

In a particular preferred embodiment, the serovar *hurstbridge* rRNA genetic sequence or a derivative, homologue or analogue thereof, is employed to identify similar genes from cells, tissues, or organ types of a host organism, in particular, the cells, tissues or organs of the urogenital tract including the bladder, uterus, fallopian tubes or kidney, or body fluids or exudates such as urine or cerebrospinal fluid, amongst others, which may be infected with a pathogenic *Leptospira* bacterium.

According to this embodiment, there is contemplated a method for identifying a related rRNA genetic sequence in a host organism which may be infected with a pathogenic *Leptospira* bacterium, said method comprising contacting cellular extract or nucleic acid sample obtained from said host organism with a hybridisation effective amount of a serovar *hurstbridge* rRNA genetic sequence, or a functional part thereof, and then detecting said hybridisation. Accordingly, this embodiment of the present invention also relates to a method of identifying a serovar of *Leptospira* which is related to leptospiral serovar *hurstbridge*, in particular the serovar deposited with AGAL on 15 November, 1995 under Accession No. N95/69684.

Said rRNA genetic sequence may be labelled with a reporter molecule which is capable of giving an identifiable signal (eg. a radioisotope such as ^{32}P or ^{35}P or a biotinylated molecule).

An alternative method contemplated in the present invention involves hybridising two nucleic acid "primer molecules" of at least 15 nucleotides in length derived from an rRNA sequence of the leptospiral serovar *hurstbridge* or its complementary sequence to a nucleic acid "template molecule" derived from a cell, tissue or organ of a host animal being tested for the presence of a pathogenic *Leptospira* bacterium, said template molecule herein defined as a related leptospiral 16S rRNA genetic sequence, or a

functional part thereof, or its complementary sequence. Specific nucleic acid molecule copies of the template molecule are amplified enzymatically in a polymerase chain reaction. Methods for the isolation of said template molecule and for the polymerase chain reaction are known to those skilled in the art.

The nucleic acid primer molecules are generally single-stranded synthetic oligonucleotides although the present invention also contemplates other primers. According to this embodiment, the nucleic acid primer molecule consists of a combination of any of the nucleotides adenine, cytidine, guanine, thymidine, or inosine, or functional analogues or derivatives thereof, capable of being corporated into a polynucleotide molecule.

Preferably, each nucleic acid primer molecule is any nucleotide sequence of at least 15 nucleotides in length derived from, or complementary to the nucleotide sequence of serovar *hurstbridge* 16S rRNA or a derivative, homologue or analogue thereof. In a particularly preferred embodiment, at least one primer molecule is substantially the same as, or complementary to, nucleotide sequences set forth in SEQ ID NOs:2 and 3.

The nucleic acid template molecule may be in a recombinant form, in a virus particle, bacteriophage particle, yeast cell, animal cell, or a plant cell. Preferably, the related genetic sequence originates from a mammalian cell, tissue or organ, optionally infected with a pathogenic leptospiral bacterium such as serovar *hurstbridge*. More preferably, said mammalian cell, tissue or organ further originates from a livestock or companion animal which is capable of being infected with said bacterium, in particular a livestock or companion animal selected from the list comprising pigs, cattle, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others..

A further aspect of the present invention provides an immunologically interactive molecule prepared against one or more immunogens of a pathogenic *Leptospira* bacterium or derivative serovar thereof which grows at temperatures of 13°C to 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml

8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

In a particularly preferred embodiment, said bacterium is the leptospiral serovar *hurstbridge* or a derivative serovar thereof. In a most particularly preferred embodiment, said bacterium is the serovar deposited with AGAL on 15 November, 1995 under AGAL Accession No. N95/69684 or a derivative serovar thereof.

The term "immunologically interactive molecule" as used herein shall be taken to include polyclonal or monoclonal antibodies, or functional derivatives thereof, for example Fabs, SCABS (single-chain antibodies) or antibodies conjugated to an enzyme, sadioactive or fluorescent tag, the only requirement being that said immunologically interactive molecule is capable of binding to an immunogen derived from or present in or present on the surface of a serovar of *Leptospira spp.*, in particular the serovar *hurstbridge*.

In an alternative embodiment, the present invention provides an immunologically interactive molecule prepared against one or more immunogens of a pathogenic *Leptospira* bacterium or derivative serovar thereof which is capable of growing at temperatures of 13°C to 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine, wherein at least one of said immunogens is a surface lipopolysaccharide molecule.

In a particularly preferred embodiment of the invention, said *Leptospira* bacterium is the serovar *hurstbridge* or a derivative serovar thereof. Most preferably, said bacterium is the serovar deposited with AGAL on 15 November, 1995 under AGAL Accession No. N95/69684 or a derivative serovar thereof.

In a related embodiment, the immunologically interactive molecule of the present invention may be prepared against an immunogen which mimics, or cross-reacts with

a B cell or T cell epitope of a surface lipopolysaccharide molecule of a pathogenic *Leptospira* bacterium, preferably the serovar *hurstbridge* or a derivative serovar thereof, more preferably the serovar deposited with AGAL on 15 November, 1995 under AGAL Accession Number N95/69684.

Conventional methods can be used to prepare the immunologically interactive molecules. For example, by using the bacterial strain or serovar of the present invention or an immunogen derived therefrom, polyclonal antisera or monoclonal antibodies can be made using standard methods. As demonstrated in Example 8 described herein, a mammal, (e.g., a mouse, hamster, or rabbit) can be immunised with an immunogenic form of a leptospiral serovar *hurstbridge* which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a serovar of serovar *hurstbridge* include conjugation to carriers or other techniques well known in the art. For example, the bacterium can be administered in the presence of adjuvant. The progress of immunisation can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies. Following immunisation, antisera can be obtained and, if desired IgG molecules corresponding to the polyclonal antibodies may be isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunised animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalising these cells and yielding hybridoma cells. Such techniques are well known in the art. For example, the hybridoma technique originally developed by Kohler and Milstein (1975) as well as other techniques such as the human B-cell hybridoma technique (Kozbor *et al.*, 1983), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, 1985), and screening of combinatorial antibody libraries (Huse *et al.*, 1989). Hybridoma cells can be screened immunochemically for production of antibodies which are specifically reactive with the polypeptide and monoclonal antibodies isolated.

As with all immunogenic compositions for eliciting antibodies, the immunogenically effective amounts of the immunogen must be determined empirically. Factors to be considered include the immunogenicity of the immunogen, whether or not it is to be complexed with or covalently attached to an adjuvant or carrier protein or other carrier and route of administration for the composition, i.e. intravenous, intramuscular, subcutaneous, *etc.*, and the number of immunising doses to be administered. Such factors are known in the vaccine art and it is well within the skill of immunologists to make such determinations without undue experimentation.

The term "antibody" as used herein, is intended to include fragments thereof which are also specifically reactive with a molecule which comprises, mimics, or cross-reacts with a B cell or T cell epitope of a surface lipopolysaccharide or a serovar of leptospiral serovar *hurstbridge*, in particular the serovar deposited with AGAL under Accession Number N95/69684. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal or fragments of antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any immunogen of leptospiral serovar *hurstbridge*, in particular the serovar deposited under AGAL Accession Number N95/69684. Preferably, said immunogen is a surface lipopolysaccharide molecule or a molecule which mimics a continuous or discontinuous B-cell or T-cell epitope of same.

The polyclonal, monoclonal or chimeric monoclonal antibodies can be used to detect the pathogenic bacterium of the invention or a derivative serovar thereof in various

biological materials, for example they can be used in an ELISA, radioimmunoassay or histochemical tests. Said antibodies are also useful in the detection of the isolated immunogen against which they are prepared, in either impure or pure form. Thus, the antibodies can be used to test for binding to the leptospiral serovar *hurstbridge*, or a derivative serovar thereof in a sample or to test for binding to the isolated immunogen or to test for binding to any molecule which cross-reacts with a B cell or T cell epitope of same.

A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilised on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time and under conditions sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule.

In this case, the first antibody is raised to an immunogen of a pathogenic *Leptospira* bacterium, wherein said bacterium is preferred to be the serovar *hurstbridge* or a serovar deposited with AGAL under Accession Number N95/69684 as described herein. More preferably, said first antibody is raised to an immunogen of said pathogenic *Leptospira*

bacterium wherein, said immunogen is a surface lipopolysaccharide of the serovar *hurstbridge*.

The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention the sample is one which might contain serovar *hurstbridge* or a derivative serovar thereof or alternatively, an immunogen of serovar *hurstbridge* or a derivative serovar thereof.

In the typical forward sandwich assay, a first antibody raised against serovar *hurstbridge* or an immunogen thereof is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking, covalent binding or physically adsorption, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes) and under suitable conditions (e.g. 25°C) to allow binding of any antigen present in the sample to the antibody. Following the incubation period, the reaction locus is washed and dried and incubated with a second antibody specific for a portion of the first antibody. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the hapten.

An alternative method involves immobilising the target molecules in the biological sample and then exposing the immobilised target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the

strength of the reporter molecule signal, a bound target may be detected by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognised, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody-hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. The term "reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in enzyme immunoassays (EIA), the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescene and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed. It will be readily apparent to the skilled technician how to vary the above assays and all such variations are encompassed by the present invention.

Accordingly, a further aspect of the present invention provides a method for the detection, identification or quantification of a pathogenic *Leptospira* bacterium or derivative serovar thereof, wherein said method comprises the steps of incubating the material or bacteria derived therefrom with an antibody which recognises said bacteria or an immunogen derived therefrom for a time and under conditions sufficient for an antibody: immunogen or antibody; bacterium complex to form and subjecting said complex to a detecting means.

According to this aspect of the invention, the complex may be detected by using the bacterium or immunogen derived therefrom or the antibody molecule with a reporter molecule attached thereto. Alternatively, the complex may be detected by the addition of a second antibody labelled with a reporter molecule.

Preferably, the invention according to this aspect provides a method for the detection, identification or quantification of a pathogenic *Leptospira* bacterium or derivative serovar thereof which is capable of growing in a medium as hereinbefore described, at

temperatures of 13°C to 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, more preferably at least 150µg/ml 8-azaguanine, even more preferably at least 200µg/ml 8-azaguanine and most preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

In a most particularly preferred embodiment, this aspect of the invention and the embodiments described therein relate to a method for the detection, identification or quantification of the leptospiral serovar *hurstbridge*, such as the serovar deposited under AGAL Accession No. N95/69684.

According to this aspect of the invention, the material or bacteria derived therefrom is in a biological tisuue or organ derived from a mammalian animal which is a host for a bacterium of the genus *Leptospira*, in particular serovar *hurstbridge* or derivative serovar thereof. Preferably, said mammalian animal is a livestock or companion animal, in particular a livestock or companion animal selected from the list comprising pigs, cattle, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

The biological sample to be tested may be any cell, tissue or organ which is capable of being infected with a bacterium of the genus *Leptospira*, in particular a cell, tissue or organ of the urogenital tract such as kidney, bladder, fallopian tube, uterus or endometrium, testes, or a body fluid or exudate such as, but not limited to urine or cerevrospinal fluid, amongst others. The present invention also contemplates the use of blood or blood-derived products as a biological sample suitable for the detection, identification or quantification of serovar *hurstbridge*.

A further aspect of the present invention contemplates a kit for the rapid and convenient assay of pathogenic *Leptospira* bacterium or derivative serovar thereof in a biological sample, wherein said bacterium is capable of growing at temperatures of 13°C to 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

In a particularly preferred embodiment, the present invention contemplates a kit for the rapid and convenient assay of a pathogenic *Leptospira* bacterium or derivative serovar thereof in a biological sample, wherein said bacterium is further characterised as leptospiral serovar *hurstbridge* or a derivative serovar thereof according to any or all of the descriptions provided herein, for example the serovar deposited under AGAL Accession No. N95/69684.

In one embodiment, said kit is compartmentalised to receive several first containers adapted to contain at least one immunogen each of the leptospiral serovar *hurstbridge* and several second containers adapted to contain an antibody molecule which binds to said pathogenic *Leptospira* bacterium, derivative serovar thereof or immunogen derived therefrom, or alternatively, said second container contains an antibody molecule which binds to serovar *hurstbridge* or a derivative serovar thereof or immunogen derived therefrom. Preferably, said second container contains an antibody which binds to the serovar deposited with AGAL under Accession Number N95/69684, or an immunogen derived therefrom, in particular a surface lipopolysaccharide immunogen.

According to this embodiment of the present invention, said antibody molecule is optionally labelled with a reporter molecule capable of producing a detectable signal as hereinbefore described. If the first antibody molecule is not labelled with a reporter molecule, the kit also provides several third containers which contain a second antibody which recognises the first antibody and is conjugated to a reporter molecule. The reporter molecule used in this kit may be an enzyme, a radio-isotope, a fluorescent molecule or bioluminescent molecule, amongst others.

When the kit contains a first antibody or second antibody molecule which is conjugated to a reporter molecule which is an enzyme, then said kit also provides several fourth containers which contain a specific molecule for said enzyme to facilitate detection of the immunogen: antibody complex or immunogen: antibody: antibody complex.

Optionally, the first, second, third and fourth containers of said kit may be colour-coded

for ease-of-use.

In an exemplified use of the subject kit, a control reaction is carried out in which the contents of the first container are contact with the contents of the second container for a time and under conditions sufficient for an immunogen:antibody complex to form in said first container. At the same time the sample to be tested is contacted with the contents of the second container for a time and under conditions sufficient for an immunogen:antibody complex to form in said second container. If the antibody of the second container provided is not labelled with a reporter molecule, then the complexes produced in said first and second containers are contacted with the antibody of the third container for a time and under conditions sufficient for a tertiary immunogen:antibody:antibody complex to form. The immunogen:antibody complex of immunogen:antibody:antibody complex is then subjected to a detecting means as hereinbefore described. In analysing the results obtained using said kit, the control reaction carried out in said first container should always provide a positive result upon which to compare the results obtained in said second container which contains the test sample.

In an alternative embodiment, the present invention contemplates a kit for the rapid and convenient assay of leptospiral serovar *hurstbridge* or a derivative serovar thereof in a biological sample, wherein said kit is compartmentalised to receive several first containers adapted to contain two non-complementary primer molecules of at least 10 nucleotides, preferably at least 20 nucleotides and more preferably, at least 22 nucleotides in length wherein the first of said primer molecules is substantially identical to a region of the nucleotide sequence set forth in any one of SEQ ID NOs:1 or 2 or 3 or a derivative, homologue or analogue thereof and the second of said primer molecules is substantially identical to the complement of a region of the sequence set forth in any one of SEQ ID NO:1 or 2 or 3 or a derivative, homologue or analogue thereof. Those skilled in the art will be aware of suitable combinations of nucleic acid primer molecules for the performance of this aspect of the present invention.

Preferably, said primer molecules are substantially the same as the primer molecules set forth in SEQ ID NOS: 2 and 3 or are at least 80% identical thereto.

According to this embodiment, said kit also contains several second containers adapted to contain a reaction mixture comprising buffer and salt solution either ready-for-use or in concentrated form and several third containers adapted to contain an enzyme suitable for use in a polymerase chain reaction, for example any heat stable DNA polymerase enzyme, in particular *Thermophilus aquaticus TaqI*, or similar enzyme. Optionally, the first, second and third containers of said kit maybe colour coded for ease-of-use.

For the purposes of this embodiment of the present invention, the biological sample may be any cell, tissue, organ, body fluid or exudate of a mammalian animal which is capable of carrying a serovar of a pathogenic *Leptospira* bacterium of the invention, serovar *hurstbridge* or a derivative serovar thereof, including for example any cell, tissue or organ of the urogenital tract, bladder, kidney, uterus, endometrium, testes or fallopian tube or a body fluid or exudate such as urine or cerebrospinal fluid, amongst others. The invention also contemplates the use of blood as a biological sample which is useful for the present purpose. Alternatively, or in addition to the foregoing examples of suitable biological samples, it is also possible to use a nucleic acid extract obtained from said cell, tissue or organ sample. Preferably, said biological sample originates from a livestock animal such as a livestock or companion animal, in particular a livestock or companion animal selected from the list comprising pigs, cattle, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

In an exemplified use of the subject kit described in this embodiment, a test sample reaction is carried out wherein the contents of the first, second and third containers are combined and a biological sample to be tested is added thereto. A negative control reaction may also be set up in which no biological sample is added to the reaction mixture. The test sample and negative control reactions are incubated for a time and under conditions sufficient for the amplification of DNA sequences which originate from the subject bacterium to occur.

A further aspect of the present invention contemplates a diagnostic test for the identification of a *Leptospira* pathogen in a biological sample using the methods, reagents and kits of the present invention as hereinbefore defined.

A still further aspect of the present invention contemplates a vaccine preparation comprising:

1. one or more immunogens which are immunologically cross-reactive with a cellular component of a pathogenic *Leptospira* bacterium or derivative serovar thereof wherein said bacterium is capable of growing at temperatures of 13°C to 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine; and
2. one or more pharmaceutically acceptable carriers, adjuvants and/or diluents.

In a preferred embodiment, the immunogen according to this aspect of the invention is immunologically cross-reactive with a bacterium characterised according to any or all of the descriptions provided herein as leptospiral serovar *hurstbridge*, in particular the leptospiral serovar deposited with AGAL under Accession Number N95/69684.

In a more preferred embodiment, at least one of said immunogens is a surface lipopolysaccharide.

According to this aspect of the present invention, the immunogen component of an effective vaccine preparation may also comprise a complete, attenuated leptospiral serovar *hurstbridge* which has been pre-treated to render it non-infectious and predominantly asymptomatic. Methods for attenuating said leptospiral serovar include, but are not limited to heat-killing, irradiation or genetic modification to remove genetic material related to pathogenesis.

The vaccine preparation of the present invention is contemplated to exhibit excellent

therapeutic activity, for example, in the prevention of diseases associated with infection by leptospiral pathogens such as serovar *hurstbridge*, in particular reproductive disease. Preferably, said vaccine preparation is effective in mediating an immune response when administered to a mammalian animal, in particular to a livestock or companion animal, such as a livestock or companion animal selected from the list comprising pigs, cattle, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

In a more preferred embodiment, said vaccine preparation induces humoral immunity against serovar *hurstbridge* when administered to said mammal.

In a most particularly preferred embodiment, said vaccine preparation induces humoral immunity against the leptospiral serovar *hurstbridge* deposited with AGAL under Accession Number N95/69684.

The present invention also extends to vaccine preparations comprising recombinant polypeptide immunogens derived from a leptospiral bacterium which may be characterised as serovar *hurstbridge* according to any or all of the embodiments described herein.

The term "mediating an immune response" as used herein is defined in its broadest context to include the elicitation of T-cell activation by an immunogen and/or the generation, by B-cells, of neutralising antibodies which cross-react with one or more molecules encoded by a pathogenic serovar of *Leptospira* as described herein, or a derivative serovar thereof. In particular, said neutralising antibodies cross-react with one or more molecules encoded by serovar *hurstbridge* or derivative serovar thereof.

The vaccine preparation may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (eg using slow release molecules). Depending on the route of administration, the immunogens contained therein may be required to be coated in a material to protect them from the action of enzymes, acids and

other natural conditions which otherwise might inactivate said immunogen. In order to administer the vaccine preparation by other than parenteral administration, they will be coated by, or administered with, a material to prevent its inactivation. For example, the immunogen may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. "Adjuvant" as used herein is to be taken in its broadest sense and includes any immune-stimulating compound such as a cytokine. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes.

The vaccine preparation of the present invention may also be administered parenterally or intraperitoneally. Dispersions of the immunogen component can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as licithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or

sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the immunogen of the present invention in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by heat-sterilisation, irradiation or other suitable sterilisation means. Generally, dispersions are prepared by incorporating the various sterilised active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When immunogens are suitably protected as described above, the protected immunogen may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral administration, the protected immunogen may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of immunogen in such vaccine preparations is such that effective immunisation will be achieved with between one and five doses of said vaccine.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic

acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the immunogen of the present invention may be incorporated into sustained-release preparations and formulations.

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutically acceptable carrier.

For the purposes of exemplification only, the present invention is further described by the following non-limiting Figures and Examples.

EXAMPLE 1

General strategy and selection of swine herds for culture

Three swine herds in Victoria and New South Wales were selected for culture, all serologically positive to *Leptospira interrogans* serovar *bratislava*. *Leptospira* bacteria had been visualised in each herd by immunofluorescent staining of tissues, despite a lack of serological evidence of infection with the known Australian pig *L.interrogans* serovars, *pomona* and *tarassovi*. Cultures were established from uterus, fallopian tube and kidney of each animal. The objective was to maximise the chance of isolating serovar *bratislava* by culturing from both baconer-age gilts and sows.

The sample size was limited to 30 cultures for each animal or less. The culture program involved the use of an initial 24 culture tubes for each animal: three tissues, four antibiotic combinations, and two dilutions.

EXAMPLE 2

Collection of tissues for culture

Swine herds were selected according to Example 1. Tissues for culture were collected from animals at the Hurstbridge abattoir (Herds A and B) and the Altona abattoir (Herd C). Bladders were tied off with cable ties at the point of removal and uterus with fallopian tubes, and kidneys, were collected in sterile bags. Blood was also collected at the point of slaughter and matched with tissue samples. Pigs were identified at the point of slaughter by individual tattoo.

EXAMPLE 3

Bacterial Cultures

Tissue samples obtained as described in the preceding Examples were processed as soon as possible to limit the degree of autolysis. Fallopian tube segments, uterus endometrial

scrapings and kidney samples were homogenised in phosphate-buffered albumin to protect leptospires then diluted to a final concentration of 1:100 prior to inoculating two 7.5 ml volumes of culture medium with one and five drops respectively. Samples were incubated at 30°C for up to 6 months and examined at intervals of approximately two to three weeks. Four different formulations of Tween 40/80 semisolid media were used, with different combinations of antibiotics, according to a matrix shown in Table 1.

Table 1

Matrix of antibiotic combinations used in the culture program designed to isolate *Leptospira interrogans* serovar *bratislava*.

LEPTOSPIRA MEDIA MATRIX

Medium	5-Fluorouracil	Rifampicin	Amphotericin B	Naladixic Acid
M1	100µg/ml	NONE	NONE	NONE
M2	200µ/ml	NONE	NONE	NONE
M3	300µ/ml	NONE	NONE	20µg/ml
M4	100µ/ml	10µg/ml	2µg/ml	NONE

Cultures were established from 27 sows and gilts, 24 of which were from the three target herds (Table 2). Leptospires were observed in six cultures derived from five animals in two of the three target herds (B and C). Isolation was achieved in five cases, as shown in Table 3.

Structures were observed in several cultures from herd A similar to non-motile leptospires and appeared typical of *bratislava* when first observed in cultures.

Table 2

Pigs from which tissues were cultured in an effort to isolate *Leptospira interrogans* serovar *bratislava*.

Pig Number	Abattoir	Type and Age of Pig	Herd of Origin	Date of Culture
1	Hurstbridge	Gilt	A	20/10/93
2	Hurstbridge	Gilt	A	26/10/93
3	Hurstbridge	Gilt	A	26/10/93
4	Hurstbridge	Gilt	A	3/11/93
5	Hurstbridge	Gilt	A	3/11/93
6	Hurstbridge	Sow	A	4/11/93
7	Hurstbridge	Sow	N.S.W. herd	4/11/93
8	Hurstbridge	Sow	A	4/11/93
9	Hurstbridge	Sow	N.S.W. herd	23/11/93
10	Hurstbridge	Sow	Victorian herd	23/11/93
11[1]	Hurstbridge	Gilt	B	2/1/94
12	Hurstbridge	Gilt	B	21/1/94
13	Hurstbridge	Gilt	B	21/1/94
14	Hurstbridge	Gilt	B	21/1/94
15	Hurstbridge	Gilt	B	21/1/94
16[2]	Hurstbridge	Sow	B	4/2/94
17[2]	Hurstbridge	Sow	B	4/2/94
18[2]	Hurstbridge	Sow	B	4/2/94
19	Hurstbridge	Sow	B	4/2/94
20	Hurstbridge	Sow	B	4/2/94
21	Hurstbridge	Sow	B	4/2/94
22	Altona	Sow	C	13/4/94
23	Altona	Sow	C	13/4/94
24[2]	Altona	Young sow	C	4/5/94
25[2]	Altona	Young sow	C	4/5/94
26	Altona	Young sow	C	4/5/94
27	Altona	Young sow	C	4/5/94

[1]Discarded early as cultures incorrectly inoculated.

[2]Leptospiral Isolates were obtained from these pigs. See Table 3.

TABLE 3

Observation of Leptospires in Cultures.

Number	Herd	Pig No.	MAT Titre to *bratislava*	Pig Type	Date Cultured	Date First Observed	Weeks of Culture	Tissue	Isolated	Remarks
1	B	16	128	Sow	4/2/94	16/2/94	2	Uterus	Yes	These isolates appear to be identical.
2	B	16	128	Sow	4/2/94	20/2/94	2	Kidney	Yes	
3	B	17	32	Sow	4/2/94	23/3/94	7	Kidney	Yes	
4	B	18	64	Sow	4/2/94	26/4/94	11	Uterus	Yes	
5	C	25	<32	Young sow	4/5/94	28/6/94	8	Uterus	No	Typical leptospires, lost in culture.
6	C	24	<32	Young sow	4/5/94	31/8/94	17	Kidney	Yes	Typical leptospires. Agglutinated by antiserum to isolate 1.

However no motile leptospires developed from these cultures and isolation was not achieved. The identity of these possible leptospires could not be confirmed.

Four isolates of an organism from three sows were obtained from herd B. Leptospires were also observed in two cultures from Herd C but only one isolate was obtained.

EXAMPLE 4

Microscopic agglutination test

The microscopic agglutination test (MAT) (Cole *et al.*, 1973) was performed using serovar *hurstbridge* isolate No. 6 (Table 3) as the live antigen. Sera were typically tested at final dilutions (including antigen) from 1/32 to 1/256 or above. The titre was expressed as the reciprocal of this dilution.

MAT titres to *bratislava* ranged from <32 to 128 in pigs from which isolates were obtained.

EXAMPLE 5

Polymerase chain reaction specific for pathogenic leptospires

PCR was based on the detection of the 16S ribosomal RNA gene, using both the oligonucleotide primers of Hookey (1992). It was found necessary to adjust the annealing temperatures used to achieve the published levels of specificity.

Samples for PCR were heated before testing, at 100°C for 10 minutes. The typical PCR reaction volume of 50 µl consisted of 1 µl sample, 5 µl buffer concentrate (giving final concentrations of 0.1M Tris-HCl, pH 9.0, O.5M KCl, 0. 1% gelatin, 15 mM $MgCl_2$, 1% Triton X-100), 5 µl dNTPs (each at final concentrations of 0.25 mM), 1 µl forward primer and 1 µl reverse primer at appropriate dilutions in water, 1 µl Taq DNA

polymerase 1/5 in diluting buffer, and 36 µl water. The enzyme diluting buffer consisted of 10 mM Tris-HCl pH 7.5, 300 mM KCl, 1 mM DTT, 0.1 mM EDTA, 500 µg/ml bovine albumin, 50% glycerol and 0.1% Triton X-100.

PCR was performed in a Perkin Elmer Cetus GeneAmp PCR System 9600. PCR conditions using the primers of Hookey (1992) were 35 cycles of 94°C (10 seconds), 59°C (10 seconds) and 72°C (10 seconds).

PCR was shown to be a reproducible method for detecting leptospires in culture. After modification of annealing conditions, PCR using the primers of Hookey (1992) detected pathogenic leptospires such as *bratislava, tarassovi* and *pomona*, but not the saprophytic *L. biflexa* serovar *patoc*. This is in accord with the published literature.

EXAMPLE 6

Gene sequencing

The sequences of the 16S ribosomal RNA genes of a number of leptospiral serovars were determined as follows. A PCR product of about 1.4 kb, corresponding to most of the gene sequence, was generated using oligonucleotide primers 27F and 1392R from conserved regions of the gene. The base numbers specified relate to the sequence of the gene from *E. coli*. The DNA product was purified using a Wizard™ PCR clean-up kit. The eluted product was then run on a 1% low-melting-temperature agarose gel. The desired band was excised using a scalpel blade and further purified using a Wizard clean-up kit.

Sequencing was then performed using overlapping forward primes (27F, 530F, 926F) and reverse primers (1392R, 1100R, 660R, 519R) so that a reliable sequence could be derived after the resolution of ambiguities. This was undertaken on a Biosystems Model 373A DNA Sequencer at the Monash University Department of Microbiology. Sequencing was attempted on the genes from serovars *bratislava, hardjo* (genotype Hardjobovis), *copenhageni, tarassovi* and *australis*. A partial gene sequence was also obtained using the 27F primer for two isolates (1 and 2) of the *Leptospira* serovar

hurstbridge cultured from Herd B.

Complete 16S ribosomal RNA gene sequences were obtained for serovars *bratislava, hardjo* (genotype Hardjobovis), *copenhageni* and *tarassovi*. These were compared with published sequences from serovars *pomona, canicola, icterohaemorrhagiae* and several others, available through GENEBANK. A partial sequence derived for *L. biflexa* serovar *patoc* corresponded to a sequence in GENEBANK.

EXAMPLE 7

Characterisation of isolates from Herds B and C

Four leptospiral isolates were obtained from Herd B. This organism was characterised according to its agglutination with antisera against a range of leptospiral pathogens, and was examined by transmission electron microscopy. It was tested by PCR using the primers described by Hookey (1992), in order to characterise it as either a pathogen or a saprophyte. It was also sent to the International Leptospirosis Reference Laboratory in Brisbane, Australia for further characterisation with respect to both agglutination properties and growth characteristics.

Isolates from herd B appeared as typical leptospires under both dark ground microscopy and transmission electron microscopy. These isolates were not agglutinated to high titre by antisera against *bratislava, pomona, tarassovi, hardjo, copenhageni* or a number of other pathogenic serovars. The herd B organism was found to autoagglutinate strongly, and the results of these agglutination experiments were therefore difficult to read.

Isolate 1 from herd B was sent to the International Leptospirosis Reference Laboratory in Brisbane, Australia for confirmation of lack of agglutination by antisera to known leptospiral pathogens. It was also demonstrated that the isolate grew persistently at 13°C, and in the presence of 8-azaguanine, implying that it was a saprophyte and not a pathogen. In contrast we found that the isolate gave a band in PCR using the primers

designed by Hookey (1992), indicating that it was a pathogen and not a saprophyte. To resolve this contradiction, a partial sequence of the 16S ribosomal RNA gene was obtained, between the bases 51 to 199. Identical sequence information was obtained for isolates 1 and 2 from herd B, using the forward primer 27F.

A sequence homology comparison between the herd B leptospire and a number of leptospiral serovars is shown in Table 4. The results of this and more detailed comparison indicate that:

a)the new isolate falls within the pathogenic grouping and not the saprophytic grouping of leptospires;

b)the new isolate nevertheless is not *bratislava, pomona* or *tarassovi*;

c)the new isolate is most similar, with respect to rRNA gene sequence identity, to *L. inadai* serovar *lyme*.

The isolate from Herd C failed to agglutinate with antisera to a number of known pathogenic leptospires (Table 5). However it agglutinated to high titre with rabbit antiserrum raised against the Herd B isolate (Table 5). This indicates that it is probably the same organism. The Herd C isolate showed no autoagglutination when first obtained, unlike the isolates from Herd B.

TABLE 4

Homology of the sequence of the region of the 16S ribosomal RNA gene from base 51 to base 199 between the leptospire isolated from herd B and a number of other serovars.

Group	Species	Serovar	Percentage Homology
Pathogens	*L. interrogans*	*bratislava*	87.6
		pomona	90.2
		canicola	88.3
	L. inadai	*lyme*	**96.6**
Saprophyte	*L. biflexa*	*patoc*	75.2

TABLE 5

Microscopic agglutination test titres given by isolate 6 from Herd C with some high titre rabbit antisera.

Rabbit Antiserum against:	**Agglutination Titre**
bratislava strain 834	<4
bratislava strain Jez	<4
pomona	32
tarassovi	64
isolate 1 from Herd B	≥8192

EXAMPLE 8

Production of a rabbit antiserum against the leptospire isolated from Herd B

Isolate 1 was grown in culture to about 10^8 organisms/ml in Kortof's (protein-free) medium. The culture was heated at 56°C for 30 minutes to kill the leptospires and emulsified with an equal volume of Montanide ISA 50 adjuvant. A rabbit was immunised weekly for six weeks with 2ml of adjuvanted leptospire, each dose being distributed over ten subcutaneous sites. Blood was obtained from the ear two weeks after the last dose.

EXAMPLE 9

PCR specific for serovar *hurstbridge*

A PCR was performed with oligonucleotide primers designed to be specific for serovar *hurstbridge* as set forth in SEQ ID NOs:2 and 3.

Cultures of five strains of serovar *hurstbridge* and of seven other leptospires tabulated below (representative of all seven pathogenic species), were grown in EMJH medium an adjusted to a concentration of 2 x 10^8 organisms/ml. DNA was extracted by the silica absorption method of Boom *et al* (1990) and during this process a volume of 100 µl of culture was reduced to 25 µl, of which 5 µl was tested in the PCR reaction. Thus 4 x 10^6 organisms were tested in each PCR reaction.

The forward oligonucleotide primer (SEQ ID NO:2) corresponded to a region of the 16S ribosomal RNA gene which differed from that of other leptospires with which it was compared. The reverse oligonucleotide primer (SEQ ID NO:3) was as designed by Hookey (1992) and is one of a pair of primers used for a PCR test specific for pathogenic leptospires.

The typical PCR reaction volume of 50 µl consisted of 5 µl sample, 5 µl of buffer concentrate giving final concentrations of 0.1M Tris-HCl, pH 9.0, 0.5M KCl, 1% Triton X-100, 20mM $MgCl_2$), 5 µl of dNTPs (each at a final concentration of 0.2 mM), 1 µl forward primer and 1 µl reverse primer at appropriate dilutions in water (each 50 pM), 5 units Taq DNA polymerase, and water to make up the volume.

PCR was performed in a Perkin Elmer Cetus GeneAmp PCR System 9600. The PCR conditions were as follows: a) one cycle of 94°C for 3 minutes, 63°C for 1.5 minutes. 72°C for 2 minutes; b) 29 cycles of 94°C for 1 minute, 63°C for 1.5 minutes, 72°C for 2 minutes; c) a further 10 minutes held at 72°C at the end of the reaction.

The PCR products were subjected to agarose electrophoresis to detect a product of the predicted size.

EXAMPLE 10

Results of a PCR Specific for Pathogenic Leptospires

All five strains of serovar *hurstbridge* were positive in this PCR, as were all known pathogenic leptospires tested. *Leptospira biflexa* serovar *patoc*, a representative saprophytic leptospire, was negative.

EXAMPLE 11

Results of PCR Specific for Serovar *hurstbridge*

Results obtained are shown in Table 6.

TABLE 6

Results of PCR Specific for Serovar *hurstbridge*

Organism Tested	PCR Result
Serovar *hurstbridge* (5 strains)	Positive
Leptospira interrogans serovar *pomona*	Negative
Leptospira borgpetersenii serovar *tarassovi*	Negative
Leptospira noguchi serovar *panama*	Negative
Leptospira kirschneri serovar *grippotyphosa*	Negative
Leptospira inadai serovar *lyme*	Negative
Leptospira weillii serovar *cellodoni*	Negative
Leptospira santarosai serovar *varela*	Negative

EXAMPLE 12

Vaccination of pigs against serovar *hurstbridge*

An vaccine containing sufficient attenuated organisms of serovar *hurstbridge* to induce an immune response in an adult sow is prepared by conventional methods, using an aluminium adjuvant and a pharmaceutically acceptable carrier. Usually, 10^7 to 10^9 organisms is sufficient for this purpose. A control vaccine is also prepared which is of identical composition, however containing no *hurstbridge* organisms.

Immediately prior to vaccination, a blood sample is taken from all animals to determine background levels of immunity to serovar *hurstbridge*. Levels of antibodies to serovar *hurstbridge* are determined using conventional immunoassays, such as ELISA techniques. Animals which have high antibody titres (i.e. titres greater than 1:100 to 1:500) to serovar *hurstbridge* prior to vaccination are removed from the trials.

The vaccine comprising attenuated serovar *hurstbridge* and the control vaccine are administered subcutaneously to two groups of 100 sows (i.e the "vaccinated group", receiving the attenuated serovar *hurstbridge* vaccine and the "control group" receiving

the control vaccine) on a commercial pig farm. Animals are given at least two injections of 2ml each, given approximately four weeks apart. Following the second injection, a further blood sample is taken from the animals to determine antibody titres to serovar *hurstbridge*. Where necessary, animals are given further injections untils significantly high titres to serovar *hurstbridge* are apparent in animals belonging to the vaccinated group.

High titre antibodies to serovar *hurstbridge* are only apparent in animals belonging to the vaccinated group, which receive the vaccine comprising attenuated serovar *hurstbridge*, but not in animals of the control group which receive the control vaccine.

EXAMPLE 13

Reproductive succcess of sows vaccinated against serovar *hurstbridge*

Sows are vaccinated against serovar *hurstbridge* essentially as described in Example 12. Sows from both the vaccinated group and control group are subsequently mated and their reproductive histories are followed for six months following vaccination, to compare reproductive success in the two groups.

The rate of return to service of the vaccinated group is significantly ($p<0.05$) less than that of the control group, indicating a lower incidence of reproductive disease in the vaccinated group. This conclusion is supported by a significantly ($p<0.05$) higher farrowing rate apparent in the vaccinated group compared to control group animals.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The

invention also includes all of the steps, features, compositions and compounds referred to in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Daratech, Pty Ltd

(ii) TITLE OF INVENTION: Novel bacterial pathogens

(iii) NUMBER OF SEQUENCES: 3

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
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(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 148 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA or RNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Leptospira
(B) SEROVAR: hurstbridge

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAACXTGCTAG TCGAGCGGGG TAGCAATACC TAGCGGCGAA CGGGTGAGTA ACACGTGGGT 60

AATCTTCCTC CGAGTCTGGG ATAACTTTCC GAAAGGAAAG CTAATACCGG ATAGTCCTGT 120

TGGATCACAA GATTTGATAG GTAAAGA 148

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA or RNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGTTGGATCA CAAGATTTGA TA 22

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA or RNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

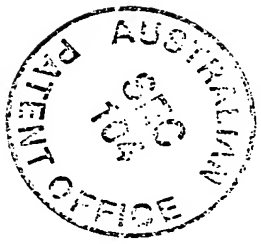
TTCACCGCTA CACCTGGAA 19

DATED this SEVENTH day of MARCH, 1997

Agriculture Victoria Services Pty Ltd.
~~Daratech Pty Ltd~~

AND

Pig Research and Development Corporation



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